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TECHNICAL MEMORANDUM 189

AN ATTEMPT TO IMPROVE THE ASSAY PRECISION OF COXIELLA BURNETII

William C. Patrick III
Jack L. Davis

DECEMBER 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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AN ATTEMPT TO IMPROVE THE ASSAY PRECISION OF *COXIELLA BURNETII*

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Product Development Division
AGENT DEVELOPMENT & ENGINEERING LABORATORIES

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December 1969

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

Limited investigations to determine the cause of assay variability associated with Coxiella burnetii contained in egg slurries showed that the type of slurry used in the assay influenced the amount of variability; moreover, the type of diluent influenced the level of assay response. Additional investigations are required to define those variables that must be controlled to increase assay precision with this organism.

DIGEST

A hypothesis was formulated to account for the erratic assay response of egg slurries containing Coxiella burnetii. The cornerstone of this hypothesis was the observation that although the control slurry assays erratically, less variability was observed among aerosol impinger samples. It was postulated that during aerosol dissemination the organism is freed of a large percentage of inert solids, and that the energy of dissemination provides individual particles with sufficient volume so that reagglomeration does not occur. Thus, particles in aerosol would tend to assay as single units and not as aggregates. This hypothesis was tested by comparing the following variables: (i) crude slurry assayed against a more purified slurry, the Biological Uniform Reference Program product; (ii) two types of diluent, heart infusion broth compared with distilled water; and (iii) the standard method of preparing the initial dilution compared with an experimental method, that is, hand shaking the initial 10^{-1} dilution versus the application of a high level of energy to an initial dilution of either 10^{-2} or 10^{-3}. The more purified slurry assayed with less variability, which was statistically significant at the 5% level of probability. Method of preparing the initial dilution was not a critical variable; however, the type of diluent was. Heart infusion broth provided higher levels of assay response than distilled water, and the difference was statistically significant at the 2.5% level of probability. These findings are considered presumptive in character rather than definitive in nature. Additional investigations are needed to define those variables that cause such erratic serological response in the test animal to C. burnetii.

I. INTRODUCTION*

A. PURPOSE

The purpose of this study was to determine if the assay precision of egg slurries containing Coxiella burnetii could be improved by the application of high levels of energy to the slurry during its dilution.

B. BACKGROUND

1. Historical Observations of the Assay

Experience has demonstrated that egg slurries that contain C. burnetii cannot be assayed with any degree of reliability. It has not been unusual to assay a common lot of slurry with a range of 2 to 5 logs among the assays when guinea pigs are the test animal. Reliability of the assay is somewhat improved by the use of mice. Not only can larger numbers of mice be used, but the serological response of the mouse seems to be more consistent than that of the guinea pig. Use of mice has improved but not eliminated the problems associated with the assay of C. burnetii, as assay variability remains in the range of 1 to 2 logs.

The variability described above pertains to "control" assays; that is, the dilution of the slurry to the appropriate level and the injection of the dilutions into the test animal. After appropriate incubation the animals are bled and tested for the presence of specific antibodies using the complement-fixation procedure.

Assay variability of impinger samples from aerosol has been of a much smaller magnitude than that of the control slurry used to form the aerosol. This observation over a period of years has led many of us to accept the 4-minute aerosol concentration, that is, the number of ID_{50} per liter of aerosol, as the only meaningful assay for any given slurry. Thus, it appears that in the process of disseminating the slurry into an aerosol and collecting the organisms in the impinger fluid, the organisms have been rendered more susceptible to assay. The most logical explanation to account for increased assay reliability via aerosol is that the organism is freed from the extraneous solids and tissues of whole egg slurry by the energy of dissemination; moreover, once freed of this debris, the dilution factor or volume of air surrounding each particle is sufficient to prevent reagglomeration. Thus, each particle tends to assay as an individual unit and not as an agglomerate. Agglomerates, while varying widely in their composition, elicit an assay response identical to that of an individual particle. In any event, this hypothesis is subject to laboratory investigation without the use of an aerosol test.

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* This report should not be used as a literature citation in material to be published in the open literature.

2. Formulation of Experimental Approach

This study is based on two experimental approaches: (i) preparing the initial dilution in a large volume of diluent (10^{-3} dilution) and subjecting this and subsequent dilutions to high levels of energy provided by different types of devices; and (ii) preparing slurries of different purity and subjecting them to different techniques of dilution.

In the first approach, the response of the initial dilution of slurry is critical. The slurry must be provided with sufficient diluent to minimize agglomeration as the organism is either released from the tissue as single units or as small particles during the application of the energy. We demonstrated in 1953 that prolonged periods of milling in the Eppenbach colloid mill do not increase the titer of C. burnetii. Probably additional organisms were released from the tissues during prolonged milling but due to the density of particles in whole egg slurry, the freshly released agent simply combined with other particulates.

The second experimental approach is based on the assumption that a more purified slurry should elicit a more uniform assay response. Purification should remove the cellular debris that interacts with itself and the organism to form aggregates that then assay as single units. Method of purification could be an important factor because one or two fractions of the total population of inert solids could be largely responsible for agglomeration. If the method of purification does not selectively remove these critical fractions, an increase in assay precision would not be observed.

II. MATERIALS AND METHODS

A. PREPARATION OF SLURRY

Five-day-old embryonated eggs were inoculated with seed via the yolk sac, incubated for 3 days, and candled for trauma; dead embryos were removed and the remaining embryos were reincubated. Embryos that died 8, 9, or 10 days postinoculation were harvested using the whole-egg technique. The egg material was homogenized into a crude slurry by blending in a Waring blender for 2 minutes, divided into 50-ml portions, and frozen. The frozen slurry was removed from storage, thawed, and assayed using procedures to be described.

B. COMPLEMENT FIXATION TEST PROCEDURE

Appropriate dilutions of slurry were inoculated intraperitoneally into 14-g mice, 0.1 ml/mouse. The mice were held for 24 days and exsanguinated; the blood was processed into sera and the sera were inactivated at 56 C for 30 minutes. The serum from each mouse was tested for the presence of specific antibody using the standard complement fixation procedure. Data from infected and noninfected mice were used to establish end points by the method of Reed and Muench (ID_{50}). The ID_{50} data were converted from 0.1 ml, the dose injected, to ID_{50}/ml.

C. SPECIFIC EXPERIMENTS

1. Experiment 1

In the first experiment, the standard method of dilution using crude slurry was compared with three experimental methods of dilution. The standard method consisted of preparing the initial dilution (10^{-1}) by the transfer of 5 ml of slurry to 45 ml of Difco sterile heart infusion broth (HIB). The dilution was shaken 25 times by hand and in this manner serial 10-fold dilutions were prepared through the 10^{-12} dilution. Dilutions of 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12} were used to inoculate mice, five mice per dilution. Three distinct dilution series were prepared to obtain an estimate of variance among the three series of dilutions. The standard dilution procedure is treatment A.

Treatment B was prepared as follows. Four milliliters of crude slurry were transferred to 4,000 ml of distilled water in the hopper of a laboratory model Eppenbach mill in order to obtain the initial dilution of 10^{-3}. This initial dilution was vigorously homogenized and recirculated in the mill at a setting of 5. The foam was allowed to settle, then subsequent 10-fold dilutions were prepared by the transfer of 5 ml of stock to 45 ml of diluent, sterile distilled water. Each dilution was shaken 25 times by hand. Inoculating dilutions and number of dilution series prepared were identical to those for treatment A.

Experimental treatment C was prepared identically to treatment B except that the distilled water for the initial dilution contained 0.1% Igepon,* a biologic-degradable detergent. Previous investigation had shown that neither Igepon nor Calgonite, when used in 0.1 or 1.0% concentrations, precipitated the egg protein at the 10^{-2} dilution; however, pH of the dilution was influenced. More specifically, 0.1 and 1.0% concentrations gave a pH of 6.45 and 7.30, respectively, for Igepon and pH of 10.0 and 11.50, respectively, for Calgonite.

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* General Aniline and Film Corp., 140 West 51st St., New York, N.Y. 10020.

Experimental treatment D was prepared by placing 1 ml of crude slurry in 99 ml of distilled water to obtain a 10^{-2} dilution. This dilution was exposed to the maximum level of ultrasonic vibration in the Biosonic device* for 3 minutes. Temperature of the dilution was maintained between 4 and 10 C by packing wet ice around the dilution blank. Subsequent dilutions were prepared for inoculation into mice as described for treatment B.

2. Experiment 2

Experiment 2 comprised a 3 by 2 factorial of those variables that appeared to be important in experiment 1. They were as follows: (i) crude whole egg slurry used in experiment 1 compared with the more refined slurry from the Biological Uniform Reference Program (BURP); (ii) standard HIB compared with distilled water as a diluent for all dilutions; (iii) method of preparing the initial dilution, that is, the standard method whereby 5 ml of slurry are transferred to 45 ml of diluent (10^{-1}) and shaken by hand compared with the Biosonic vibration of the 10^{-2} dilution as described in experiment 1. The interaction of these variables provides the eight treatments described in Table 1. Injection of mice, incubation, and testing of sera by the complement fixation procedure were identical to those procedures described in experiment 1. Three distinct dilutions were prepared for each treatment to compare variability within treatment with variability between treatments.

III. RESULTS

The first experiment provided results (Table 2) that suggested that the three experimental methods for preparing the initial dilution were superior to the standard procedure. There was more uniformity in the assay response within each experimental treatment than within the standard treatment. The F test for equality of variance did not show variability to be significant between the control and experimental treatments at the 5% level of probability. This was caused by having only 2 degrees of freedom for each treatment. It is believed that additional testing of each treatment would have shown the variance to be significantly different between control and experimental treatments.

The raw data from experiment 2 are summarized in Table 3. An analysis of variance was made on the data produced by the interaction of the three major variables (Table 4). The analysis of variance shows that the type of slurry does contribute to assay variability. The crude slurry has a variance of 0.879, whereas the refined slurry has a variance of 0.060.

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* Heat Systems-Ultrasonics, Inc., Plainview, L.I., N.Y.

TABLE 1. INTERACTION OF VARIABLES INVESTIGATED IN EXPERIMENT 2

Variable	Treatment							
	1	2	3	4	5	6	7	8
Type slurry[a/]	Crude	Crude	Crude	Crude	Ref.	Ref.	Ref.	Ref.
Diluent	HIB	HIB	Water	Water	HIB	HIB	Water	Water
Initial dilution[b/]	Std.	Exp.	Std.	Exp.	Std.	Exp.	Std.	Exp.

a. Crude slurry: Prepared by milling the entire contents of infected eggs with no additional processing. Total dry solids content of this slurry is 25%.

Reference slurry from BURP: The harvest is diluted 1 to 4 with distilled water, 2% F-20 silica added to adsorb extraneous solids, milled, then centrifuged. The supernatant liquid is filtered. This slurry has a total dry solids content of 3.5%.

b. Standard: Initial 10^{-1} dilution prepared by transfer of 5 ml of slurry to 45 ml HIB, hand shaken 25 times. All other dilutions similarly prepared.

Experimental: Initial 10^{-2} dilution prepared by Biosonic vibration (3 min) of 1 ml of slurry in 99 ml of diluent. All other dilutions prepared by transfer of 5 ml of stock to 45 ml of diluent and hand shaken 25 times.

TABLE 2. INFLUENCE OF METHOD OF DILUTION ON ASSAY RESPONSE OF CRUDE SLURRIES CONTAINING COXIELLA BURNETII

Treatment	Description		Log_{10} $MIPID_{50}/ml$[a]
A	Standard procedure: Initial dilution of 10^{-1} prepared by transfer of 5-ml stocks to 45 ml HIB. Hand shaken 25 times.		10.36
			9.67
			11.10
		Mean	11.38
		Variance	0.511
		95% Confidence	±1.54
B	Initial dilution of 10^{-3} prepared by homogenizing 4 ml stock in 4,000 ml distilled water in Eppenbach mill. Other dilutions prepared by transfer of 5 ml stock into 45 ml water and hand shaking 25 times.		9.51
			9.00
			9.16
		Mean	9.22
		Variance	0.068
		95% Confidence	±0.52
C	Same as B except that 4,000 ml contained 0.1% Igepon.		10.15
			10.00
			9.62
		Mean	9.92
		Variance	0.074
		95% Confidence	±0.54
D	Initial dilution of 10^{-2} prepared by Biosonic vibration of 1 ml stock in 99 ml distilled water. Other dilutions prepared by transfer of 5 ml stock into 45 ml water and hand shaking 25 times.		9.21
			9.66
			9.30
		Mean	9.39
		Variance	0.056
		95% Confidence	±0.47

a. Three replicates per treatment.

This difference in variability is significant at the 5% level of probability. There was no statistical difference between the means. The type of diluent employed during the assay procedure does not contribute to assay variability per se. The type of diluent does influence the level of assay response. HIB resulted in a higher mean than distilled water, and this difference was significant at the 2.5% level of probability. Method of preparing the initial dilution did not produce a significant difference, either with respect to level of assay or the amount of variance. This result was unexpected because the hypothesis described earlier was based on dilution having a profound effect on assay variability.

This experiment has shown that a more refined slurry produces a more uniform serological response in mice than does the crude slurry. This information tends to support the hypothesis that large aggregates available in the crude slurry elicit a single response in the test animal on one occasion and multiple responses on another occasion. During the last few years, personnel in our laboratory and in Applied Aerobiology Division have observed that more uniform assays are being obtained with current slurries. One explanation to account for this increased assay precision is that current programs use more refined slurries than those available 10 years ago.

A logical sequel to this experiment would be to prepare several highly purified slurries by different techniques and to assay them against the current standard, the BURP reference. It must be remembered that the BURP reference, while being significantly more purified than crude whole egg slurry, does not represent the ultimate in purification. Slurries purified by differential centrifugation or by polymer partitioning using dextran sulfate - calcium chloride precipitation represent an order of purity above the BURP reference as the BURP reference represents an order of purity over whole egg slurry.

The findings of this study may be considered "presumptive" rather than definitive. Additional investigation is required to elucidate those variables that cause significant shifts in the response of animals challenged with C. burnetii. The complement fixation procedure, as a test technique, cannot be faulted. The technique simply reflects the serological response of the animal to the antigen challenge. The serological response of the animal to the antigen is the area that must be improved if the basic assay procedure is to be improved.

TABLE 3. DEFINITION OF INDIVIDUAL TREATMENTS

	Treatment							
	1	2	3	4	5	6	7	8
Slurry	Crude	Crude	Crude	Crude	Ref.	Ref.	Ref.	Ref.
Diluent	HIB	HIB	Water	Water	HIB	HIB	Water	Water
Dilution	Std.	Exp.	Std.	Exp.	Std.	Exp.	Std.	Exp.
Assay, $\log_{10}$ $MIPID_{50}$/ml								
1	10.50	10.50	9.25	10.00	9.50	10.15	9.39	9.64
2	10.50	10.29	9.74	9.39	9.64	9.84	9.50	9.58
3	9.60	9.47	9.86	9.00	9.15	9.69	9.75	9.50
Mean	10.20	10.09	9.62	9.46	9.43	9.89	9.55	9.57

TABLE 4. ANALYSIS OF VARIANCE OF INTERACTION OF MAJOR VARIABLES

	Log_{10} MIPID$_{50}$ per ml					
	Slurry		Diluent		Dilution	
	Crude versus Ref.		HIB versus Water		Std. versus Exp.	
	10.50	9.50	10.50	9.25	10.50	10.50
	10.50	9.64	10.50	9.74	10.50	10.29
	9.60	9.15	9.60	9.86	9.60	9.47
	10.50	10.15	10.50	10.00	9.25	10.00
	10.29	9.84	10.29	9.39	9.74	9.39
	9.47	9.69	9.47	9.00	9.86	9.00
	9.25	9.39	9.50	9.39	9.50	10.15
	9.74	9.50	9.64	9.50	9.64	9.84
	9.86	9.75	9.15	9.75	9.15	9.69
	10.00	9.64	10.15	9.64	9.39	9.64
	9.39	9.58	9.84	9.58	9.50	9.58
	9.00	9.50	9.69	9.50	9.75	9.50
Mean	9.80	9.61	9.90	9.55	9.70	9.75
Variance	0.879	0.060	0.218	0.075	0.182	0.178
Significance between:						
Means	No		Yes; 2.5%		No	
Variance	Yes; 5%		No		No	

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13. ABSTRACT

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